

**REMARKS**

**1. Status of the Application**

Claims 7-24 are pending in the present application. Claims 7, 10, 13, 16, 19 and 22 have been amended. Unless otherwise noted, these claims have been amended without acquiescing to Examiner's arguments, and solely for the purpose of expediting the patent application process in a manner consistent with the PTO's Patent Business Goals (PBG)<sup>1</sup>, and without waiving the right to prosecute the unamended (or similar) claims in another application. The amendments are not intended to narrow the scope of the Claim within the meaning of *Festo*<sup>2</sup>.

The following rejections are at issue and are set forth by number in the order in which they are addressed:

- 1) Claims 7-24 are rejected under 35 U.S.C. §103(a), as allegedly obvious over Cain et al. (WO 97/18320); and
- 2) Claims 7-24 are rejected under 35 U.S.C. §103(a), as allegedly obvious over Pariza et al. (U.S. Pat. No. 5,856,149).

A *prima facie* case of obviousness requires the Examiner to cite a combination of references which (a) disclose the elements of the claimed invention, (b) suggests or motivates one of skill in the art to combine those elements to yield the claimed combination, and (c) provides a reasonable expectation of success should the claimed combination be carried out. Failure to establish any one of the these three requirements precludes a finding of a *prima facie* case of obviousness, and, without more, entitles Applicant to allowance of the claims in issue.<sup>3</sup> In addressing this rejection, Applicants focus on the independent claims since non-obviousness of an independent claim necessarily leads to non-obviousness of claims dependent

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<sup>1</sup> 65 Fed. Reg. 54603 (September 8, 2000).

<sup>2</sup> *Festo Corp. v. Shoketsu Kinzoku Kogyo Kabushiki Co.*, No. 95-1066, 2000 WL 1753646 (Fed. Cir. 2000).

<sup>3</sup> See, e.g., *Northern Telecom Inc. v. Datapoint Corp.*, 15 USPQ2d 1321, 1323 (Fed. Cir. 1990).

therefrom.<sup>4</sup> Applicants respectfully note that neither of the references teaches each element of the invention as claimed.

**1. Cain et al. Does Not Teach Each Element of the Claims**

Claims 7-24 are rejected under 35 U.S.C. §103(a), as allegedly obvious over Cain et al. (WO 97/18320). The Examiner states that Cain et al. teaches a conjugated linoleic acid composition "of which 49.7% was the cis 9, trans 11-isomer and 50.3% was the trans 10, cis 12-isomer." (Office Action, page 3). The Examiner then goes on to admit that "Cain et al. do not specifically discuss peak area percentages of the isomers of 11,13-octadecadienoic and 8,10-octadecadienoic acid, it would have been obvious to one having ordinary skill in the art that these isomers would be less than 1% of a peak area because Cain et al. teach of a conjugated linoleic acid composition product of which 49.7% was the cis 9, trans 11-isomer and 50.3% was the trans 10, cis 12-isomer. Accordingly Cain et al. teach of a composition which contains a total of 100% of both the isomers of cis 9, trans 11 isomer and which would obviously exclude the isomers of 11,13-octadecadienoic and 8,10-octadecadienoic acid." (Office Action, pages 3-4).

Applicants respectfully disagree. One of skill in the art would recognize that the composition obtained by Cain et al. **necessarily** contains other isomers than the 9,11 and 10, 12 isomers of CLA. In fact, no chemical isomerization method has been described in the literature to date which does not produce a variety of isomers. Indeed, it is impossible to isomerize linoleic acid by the methods described in Cain without producing other isomers. Accordingly, it appears that Cain et al. simply failed to conduct an analysis for the other isomers present in the isomerized product.

This conclusion is supported by reference to Sugano et al., *Lipids* 33(5):521-527 (1998)(reference 53 in Form 1449 filed November 14, 2000, copy attached hereto for the Examiner's convenience). Sugano et al. isomerized linoleic acid conditions similar to those described by Cain et al. The conditions utilized in the two references are compared in following Table.

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<sup>4</sup> §MPEP 2143.03.

Cain et al.	Sugano et al.
50 g linoleic acid, 95% pure	50 g linoleic acid, 99% pure
Solvent: 290 grams ethylene glycol	Solvent: 290 grams ethylene glycol
Catalyst: 15 g NaOH	Catalyst: 15 g NaOH
Reaction time: 2 hours	Reaction time: 2 hours
Reaction temperature: 180° C	Reaction temperature: 180° C
Reaction atmosphere: Inert	Reaction atmosphere: Nitrogen

As can be seen, the reaction conditions were almost identical. However, the results are not. As noted on page 522 of Sugano, the resulting CLA preparation contained the following CLA isomers: 29.8% c9,t11/t9,c11; 29.6% t10,c12; 1.3% c9,c11; 1.4% c10,c12; 18.6% t9,t11/t10,t12; 5.6% linoleic acid; and 13.7% other isomers. In contrast to the simplified analysis presented in Cain et al., isomerization of CLA results in the production of many different isomers, not just the desired c9,t11 and t10,c12 isomers.

As further support for this fact, the Examiner's attention is respectfully directed to examples 1-4 of the instant application. These examples compare non-aqueous alkali isomerization under high or low temperatures and aqueous alkali isomerization under high or low temperatures. **The important fact to note is that each reaction, even the low temperature non-aqueous alkali isomerization reaction (Example 1, Table 6), produced a distribution of the expected isomers, not just the c9,t11 and t10,c12 isomers.**

Thus, ample evidence supports the conclusion that the analysis of Cain et al. is either incorrect or incomplete. Cain et al. appear to have either not analyzed for the isomers or chosen not to present data pertaining to the other isomers in their analysis. This is understandable because at the time, the other isomers were not expected to have a biological effect. However, just because data on these isomers was not presented does not mean that they are not present. Indeed, the evidence establishes that they were necessarily present as a result of the reaction conditions used by Cain et al. Therefore, Cain et al. does not teach each element of the claims. A *prima facie* case of obviousness has not been established.

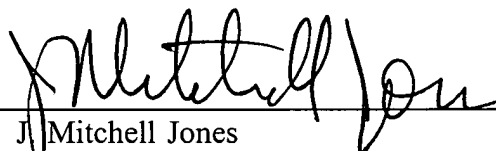
**2. Pariza et al. Does Not Teach Each Element of the Claims**

Claims 7-24 are rejected under 35 U.S.C. §103(a), as allegedly obvious over Pariza et al. (U.S. Pat. No. 5,856,149). The claims as currently pending require that the composition contain 10,12-octadecadienoic acid. Pariza et al. specifically teach at column 4, lines 36-39 that their composition does not contain the t10,c12 isomer. Thus, Pariza does not teach each element of the claims. A *prima facie* case of obviousness has not been established.

**Conclusion**

All grounds of rejection and objection of the Office Action of February 26, 2003 having been addressed, reconsideration of the application is respectfully requested. It is respectfully submitted that the invention as claimed fully meets all requirements and that the claims are worthy of allowance. Should the Examiner believe that a telephone interview would aid in the prosecution of this application, Applicant encourages the Examiner to call the undersigned collect at (608) 218-6900.

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**APPENDIX I**  
**MARKED-UP VERSION OF REWRITTEN, ADDED,**  
**AND/OR CANCELLED CLAIMS**

The following is a version of the claims pursuant to 37 C.F.R. §1.121 (c)(1)(ii) with markings showing changes made herein to the previous version of record of the claims.

**IN THE CLAIMS:**

7. (Amended once) An isomerized conjugated linoleic acid composition comprising at least fifty percent 9,11-octadecadienoic acid and 10,12-octadecadienoic acid [conjugated linoleic acid], said isomerized linoleic acid composition being characterized in including less than one percent 11,13-octadecadienoic and 8,10-octadecadienoic acid isomers expressed as peak area percentage.

10. (Amended once) An isomerized conjugated linoleic acid composition comprising at least fifty percent 9,11-octadecadienoic acid and 10,12-octadecadienoic acid [conjugated linoleic acid], said isomerized linoleic acid composition being characterized in including less than one percent 11,13-octadecadienoic and 8,10-octadecadienoic, and trans-trans-octadecadienoic acid isomers expressed as peak area percentage.

13. (Amended once) A food product comprising isomerized conjugated linoleic acid, said isomerized linoleic acid composition being characterized in including at least 50% 9,11-octadecadienoic and 10,12-octadecadienoic acid isomers and less than one percent 11,13-octadecadienoic and 8,10-octadecadienoic acid isomers expressed as peak area percentage.

16. (Amended once) A food supplement comprising isomerized conjugated linoleic acid, said isomerized linoleic acid composition being characterized in including at least 50% 9,11-octadecadienoic and 10,12-octadecadienoic acid isomers and less than one percent 11,13-octadecadienoic and 8,10-octadecadienoic acid isomers expressed as peak area percentage.

19. (Amended once) An isomerized conjugated linoleic acid composition comprising at least fifty percent conjugated linoleic acid, said isomerized linoleic acid composition being characterized in including at least 50% 9,11-octadecadienoic and 10,12-octadecadienoic acid isomers and less than one percent 11,13-octadecadienoic or 8,10-octadecadienoic acid isomers expressed as peak area percentage.

22. (Amended once) A food product comprising isomerized conjugated linoleic acid, said isomerized linoleic acid composition being characterized in including at least 50% 9,11-octadecadienoic and 10,12-octadecadienoic acid isomers and less than one percent 11,13-octadecadienoic or 8,10-octadecadienoic acid isomers expressed as peak area percentage.

# Conjugated Linoleic Acid Modulates Tissue Levels of Chemical Mediators and Immunoglobulins in Rats

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**ABSTRACT:** The effects of conjugated linoleic acid (CLA) on the levels of chemical mediators in peritoneal exudate cells, spleen and lung, and the concentration of immunoglobulins in mesenteric lymph node and splenic lymphocytes and in serum were examined in rats. After feeding diets containing either 0 (control), 0.5 or 1.0% CLA for 3 wk, there was a trend toward a reduction in the release of leukotriene B<sub>4</sub> (LTB<sub>4</sub>) from the exudate cells in response to the dietary CLA levels. However, CLA did not appear to affect the release of histamine. A similar dose-response pattern also was observed in splenic LTB<sub>4</sub>, lung LTC<sub>4</sub>, and serum prostaglandin E<sub>2</sub> levels, and the differences in these indices between the control and 1.0% CLA groups were all statistically significant. The reduction by CLA of the proportions of n-6 polyunsaturated fatty acids in peritoneal exudate cells and splenic lymphocyte total lipids seems to be responsible at least in part for the reduced eicosanoid levels. Splenic levels of immunoglobulin A (IgA), IgG, and IgM increased while those of IgE decreased significantly in animals fed the 1.0% CLA diet. This was reflected in the serum levels of immunoglobulins. The levels of IgA, IgG, and IgM in mesenteric lymph node lymphocytes increased in a dose-dependent manner, while IgE was reduced in those fed the higher CLA intake. However, no differences were seen in the proportion of T-lymphocyte subsets of mesenteric lymph node. These results support the view that CLA mitigates the food-induced allergic reaction.  
*Lipids* 33, 521-527 (1998).

Conjugated linoleic acid (conjugated derivatives of linoleic acid, CLA) exerts diverse physiological effects most of which are favorable to human health. A range of studies has shown a marked alleviating effect of CLA on mammary carcinogenesis (1-4). The mechanism underlying this effect is not yet well understood (5), but continued intake of CLA is not necessarily required for suppression of carcinogenesis (6,7). When considering the diverse effects of CLA, it is reasonable that eicosanoids are involved in the mechanism. The influ-

ence of CLA on the metabolic processes leading from linoleic acid to arachidonic acid and, hence, eicosanoids appears to be related to their desirable effects, since CLA tended to reduce the tissue level of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), a putative candidate for a cancer-promoting effect of dietary n-6 polyunsaturated fatty acids (PUFA) (8). In addition, there is a possibility that CLA itself serves as substrate of enzymatic systems for eicosanoid production, as it is shown to undergo desaturation and elongation similar to linoleic acid (9), although it is unknown whether these metabolites could be converted to eicosanoids.

Since the food allergic reaction can readily be modified by the type of dietary PUFA, either n-6 or n-3 (10,11), it is interesting to know if CLA could modify it. The clinical symptom of food allergy is induced by the production of chemical mediators such as histamine and leukotriene (LT) and PG triggered by allergen-specific immunoglobulin (Ig)E (12,13). Our previous studies showed a reduction by CLA of the serum PGE<sub>2</sub> level (8), which is one of the typical chemical mediators in the allergic reaction (12,13). In this context, Belury and Kempa-Steczko (14) showed that CLA reduces the proportion of linoleic acid dose-dependently in hepatic phospholipid and suggested this may result in modified arachidonate-derived eicosanoid production by extrahepatic tissues. More recently, Wong *et al.* (15) reported that CLA modulates certain aspects of the immune defense such as lymphocyte proliferation in mice, although the effect was not always reproduced possibly because of the dependence on the duration of the feeding period. In the present study, we measured the production of chemical mediators and the level of Ig in rats fed different levels of CLA, either 0.5 or 1.0%.

## MATERIALS AND METHODS

**Preparation of CLA.** CLA was prepared according to the method described by Ip *et al.* (16). In brief, 50 g of linoleic acid, purity >99% (Sigma Chemical Co., St. Louis, MO) was dissolved in 290 g of ethylene glycol containing 15 g of NaOH and heated at 180°C for 2 h under nitrogen. After cooling to room temperature, the content was adjusted to pH 4 and extracted with *n*-hexane. The hexane layer was washed with 5% NaCl, dehydrated with 3-A molecular sieves (Nacalai

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Abbreviations: CLA, conjugated linoleic acid; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; LT, leukotriene; MLN, mesenteric lymph node; PEC, peritoneal exudate cells; PG, prostaglandin; POD, peroxidase; PUFA, polyunsaturated fatty acid.

Tesqu, Kyoto, Japan) and dried in a rotary evaporator under nitrogen. The purity of CLA was measured by gas-liquid chromatography (Shimadzu GC-17A, Kyoto, Japan) using a Supelcowax 10 column (0.32 mm  $\times$  60 m, film thickness, 0.25  $\mu$ m; Supelco Inc., Bellefonte, PA). Column temperature was raised from 150 to 220°C at a rate of 4°C/min. The identification of peaks was carried out by the equivalent chain length method (17) and gas chromatography-mass spectrometry (Jeol Auto MS 50, Tokyo, Japan). The purity of CLA preparation was 80.7% with the following composition in percentage: 9c,11t/9t,11c, 29.8; 10t,12c, 29.6; 9c,11c, 1.3; 10c,12c, 1.4; 9t,11t/10t,12t, 18.6; linoleic acid, 5.6; and others, 13.7.

**Animals and diets.** The animal experiment adhered to the Kyushu University guidelines for the care and use of laboratory animals. Male, 4-wk-old Sprague-Dawley rats were obtained from Japan Charles River (Atsugi, Japan) and housed individually in a room with controlled temperature and light (20–23°C and lights on 0800–2000 h). After acclimation for 4 d, rats were divided into three groups of 10 rats which were given free access to the experimental diets. The diets were prepared according to the recommendation of the American Institute of Nutrition (AIN-93G diet) (18). The basal diet contained the following ingredients, in g/100 g diet: cornstarch 39.8; casein, 20.0; dextrinized cornstarch, 13.2; sucrose, 10.0; soybean oil, 7.0; AIN-93 mineral mixture, 3.5; AIN-93 vitamin mixture, 1.0; L-cystine, 0.3; choline bitartrate, 0.25; cellulose, 5.0; *tert*-butylhydroquinone, 0.002; and either linoleic acid, 1.0; linoleic acid (Control) and CLA, 0.5 and 0.5; or CLA, 1.0. Thus, LA or CLA was added at the expense of soybean oil in the AIN-93G diet. The fatty acid composition calculated from the composition of individual oils is given in Table 1. Body weight and food intake were recorded every other day. After 3 wk of feeding, five rats were used for collection of the exudate cells and the remaining five rats for other analyses. Blood was withdrawn from the abdominal aorta under light diethyl ether anesthesia and tissues were immediately excised.

**Preparation of peritoneal exudate cells (PEC).** The method described by Matsuo *et al.* (19) was adopted for the preparation of PEC. Tyrode buffer, consisting of 137 mM NaCl, 2.7 mM KCl, 0.4 mM  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 1 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 12 mM  $\text{NaHCO}_3$ , 1.8 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 5.6

mM D-glucose and 0.1% bovine serum fraction V (Boehringer Mannheim GmbH, Mannheim, Germany), pH 7.4, was injected into the rat peritoneal cavity (6 mL/100 g body weight), and the abdomen was gently massaged for 2 min. Then, the cavity was opened, and the buffer containing PEC was recovered with a plastic pipet. The fluid was centrifuged at  $200 \times g$  for 5 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in Tyrode buffer.

**Measurement of leukotriene  $B_4$  ( $\text{LTB}_4$ ) and histamine.**  $\text{LTB}_4$  was measured as described elsewhere (20–22). PEC ( $2 \times 10^6$  cells) were suspended in Tyrode buffer containing 5 mM calcium ionophore A23187 (Sigma Chemical Co.). After incubating for 20 min at 37°C, 50 mL of the acetonitrile/methanol mixture (6:5, vol/vol) and 50 ng of  $\text{PGB}_2$  (Sigma Chemical Co.), as the internal standard, were added. The mixture was kept at –20°C for 30 min and then centrifuged at  $1,000 \times g$  for 10 min. The supernatant was filtered through a 4-GV 0.22  $\mu$ m filter (Millipore Corp., Tokyo, Japan).  $\text{LTB}_4$  was measured by reversed-phase high-performance liquid chromatography (HPLC) (SCL-10A; Shimadzu Co., Kyoto, Japan) equipped with an ODS-A column (150  $\times$  6.0 mm, 5  $\mu$ L particle size; YMC, Kyoto, Japan). A mixture of acetonitrile/methanol/water (30:25:45, by vol) containing 5 mM  $\text{CH}_3\text{COONH}_4$  and 1 mM disodium EDTA, pH 5.6, was used as a mobile phase with a flow rate of 1.1 mL/min.  $\text{LTB}_4$  and  $\text{PGB}_2$  were detected by absorbance at 280 nm (SPD-10A; Shimadzu Co.). Quantitation of  $\text{LTB}_4$  was achieved by comparing the peak area of  $\text{LTB}_4$  with that of  $\text{PGB}_2$ . Histamine content in the culture supernatant was measured fluorometrically (19,23). The intracellular content of histamine also was measured after disrupting the cells by sonication.

**Preparation of spleen and mesenteric lymph node (MLN) lymphocytes.** Spleen and MLN were excised immediately after withdrawing blood from the aorta, and the tissues were immersed in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) (24,25). The lymphocytes were rinsed three times with the RPMI 1640 medium and filtered to remove tissue scum. To remove fibroblasts, cell suspension was incubated for 30 min at 37°C. Then, 5 mL of the resulting cell suspension was layered on 4 mL of Lympholyte-Rat (Cedarlane, Hornby, Canada) and centrifuged at  $1,500 \times g$  for 30 min. The lymphocyte band at the interface was recovered, and the cells were rinsed again. The lymphocytes were cultured in 10% fetal bovine serum (Intergen, Purchase, NY) in RPMI 1640 medium at a cell density of  $2.5 \times 10^6$  cells/mL with or without 2.5  $\mu$ g/mL of lipopolysaccharide (Bacto lipopolysaccharide B, *Escherichia coli* 026:B6; Difco Laboratories, Detroit, MI). After incubation at 37°C for 24 and 72 h, the concentrations of IgA, IgG, IgM, and IgE were measured by an enzyme-linked immunosorbent assay (ELISA) (26).

**T-cell population analysis.** Spleen and MLN lymphocytes were analyzed as  $\text{CD4}^+$ - and  $\text{CD8}^+$ -cells by using fluorescein-labeled mouse anti-CD4 (W3/25, mouse IgG1) or phycoerythrin-labeled mouse anti-CD8 (MRC OX-8, mouse IgG1) (both from Serotec Ltd., Kidlington, Oxford, United King-

TABLE 1  
Fatty Acid Composition of Dietary Fat<sup>a</sup>

Fatty acid	Group		
	Control	0.5% CLA	1.0% CLA
16:0	9.1	9.0	9.0
18:0	3.2	3.42	3.2
18:1	20.4	20.3	20.1
CLA	—	6.4	12.9
18:2	59.7	53.6	47.4
18:3	7.5	7.5	7.4

<sup>a</sup>Fatty acid composition was calculated from the composition of individual component fats, soybean oil, linoleic acid, and conjugated linoleic acid (CLA).



dom) (23,25). The stained lymphocytes were fixed with 2% paraformaldehyde and analyzed with the EPICS Profile II flowcytometer (Coulter Electronics Ltd., Luton, United Kingdom).

**Measurement of serum and culture supernatant Ig by ELISA.** Measurements of total Ig were executed using sandwich ELISA methods (24,25). Goat anti-rat IgE, rabbit anti-rat IgG (Fab')<sub>2</sub>, goat anti-rat IgM (all from Biosoft, Paris, France), and mouse anti-rat IgA (Zymed Lab, San Francisco, CA) were used to fix respective Ig. These antibodies were diluted 1,000 times with 50 mM carbonate-bicarbonate buffer (pH 9.6), and each well of 96-well plates was treated with 100  $\mu$ L of the solution for 1 h (2 h for IgA) at 37°C. After blocking with 300  $\mu$ L of the blocking solution overnight at 4°C, each well was treated with 100  $\mu$ L of the diluted serum or culture supernatant for 1 h (2 h for IgA) at 37°C. Bound IgA was detected by reacting stepwise with 100  $\mu$ L of peroxidase (POD)-conjugated rabbit anti-rat IgA (1,000 times dilution; Zymed) at 37°C for 2 h, IgG with 100  $\mu$ L of POD-conjugated rabbit anti-rat IgG (Fab')<sub>2</sub> (2,000 times dilution; Cappel, West Chester, PA), and IgM with 100  $\mu$ L of POD-conjugated goat anti-rat IgM (1,000 times dilution, Cappel) at 37°C for 1 h. Wells were rinsed three times with Tween-20 in phosphate-buffered saline between each step. After incubation at 37°C for 15 min with 100  $\mu$ L of 1.5% oxalic acid, absorbance at 415 nm was measured with an MPR-A4i ELISA reader (Tosoh, Tokyo, Japan). The bound IgE was detected by reacting with biotin-conjugated mouse anti-rat IgE (2,000 times dilution; Beryll, Montgomery, TX) followed by POD-conjugated avidin (5,000 times dilution, Zymed Lab) at 37°C for 1 h.

**Statistical analysis.** Data were analyzed by one-way analysis of variance followed by Duncan's new multiple-range test to identify significant differences (27). Values in the text are means  $\pm$  SE.

## RESULTS

**Growth performance and tissue weight.** As shown in Table 2, there was no difference in food intake and growth of rats for 3 wk among the groups. Thus, the feed efficiency also was comparable among the groups (mean values 0.41 to 0.42). Among tissues weighed, there was a tendency of increasing liver weight and decreasing perirenal adipose tissue weight by dietary CLA and the difference between the linoleic acid and 1.0% CLA groups was significant.

**Release of chemical mediators from PEC.** PEC isolated from rats fed linoleic acid or CLA were incubated with or without calcium ionophore A23187, and the concentrations of histamine and LTB<sub>4</sub> were measured in the medium. The content of histamine in the cells also was measured to estimate the cellular histamine contents. As shown in Figure 1, the effect of CLA on the release of histamine in PEC was diverse, and there was no significant difference in any of the parameters measured. However, the amounts of histamine stored in the cells tended to decrease with an increasing dietary level of CLA. There was a trend toward a reduction in

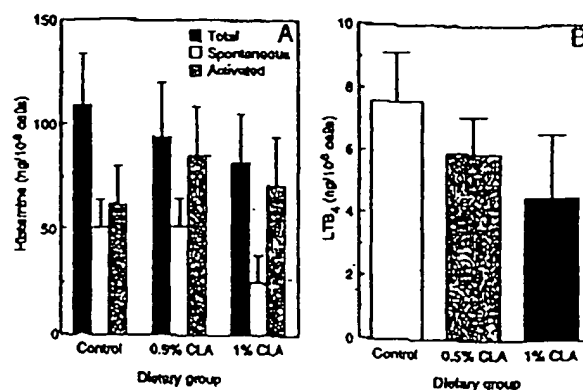


FIG. 1. Effect of dietary conjugated linoleic acid (CLA) on histamine content and release (A) and leukotriene B<sub>4</sub> (LTB<sub>4</sub>) release (B) in rat peritoneal exudate cells. Means  $\pm$  SE of five rats. Histamine release was measured in the presence and absence of calcium ionophore A23187. Total, total amounts of histamine in the cells; Spontaneous, the amount of histamine released during incubation without calcium ionophore A23187; Activated, the amount of histamine which was released from the cells when treated with A23187.

LTB<sub>4</sub> release in response to the dietary level of CLA, but the difference was not significant.

**Tissue eicosanoid levels.** The effect of CLA on LTB<sub>4</sub> and LTC<sub>4</sub> levels of spleen and lung is shown in Figures 2 and 3, respectively. CLA dose-dependently reduced the level of splenic LTB<sub>4</sub>, and the difference between the control and 1% CLA groups was significant. No effect of CLA on the splenic LTC<sub>4</sub> level was observed. However, the concentration of LTC<sub>4</sub> in lung was reduced significantly by CLA even at the 0.5% dietary level. A trend of the dose-dependent reduction of LTB<sub>4</sub> also was observed, but the difference was not significant. The results of the levels of spleen and serum PGE<sub>2</sub> are summarized in Figure 4. CLA significantly reduced the concentration of serum PGE<sub>2</sub>, while there was no effect of CLA on the splenic level of PGE<sub>2</sub>.

**Fatty acid compositions of PEC and splenic lymphocyte lipids.** The PUFA composition of PEC and splenic lympho-

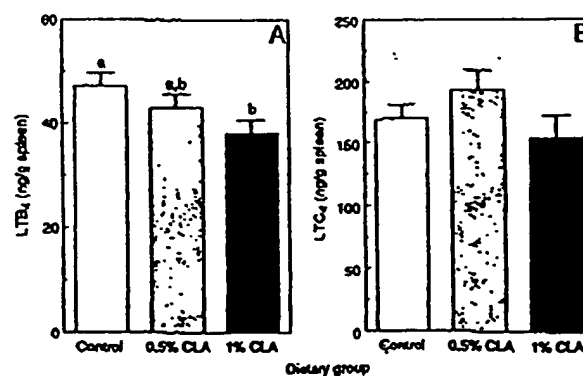


FIG. 2. Effect of dietary CLA on the concentration of splenic (A) LTB<sub>4</sub> and (B) leukotriene C<sub>4</sub> (LTC<sub>4</sub>). Mean  $\pm$  SE of five rats. Values without a common letter are significantly different at  $P < 0.05$ . For abbreviations see Figure 1.

TABLE 2  
Effects of CLA on Growth and Tissue Weights of Rats<sup>a</sup>

Parameter	Group		
	Control	0.5% CLA	1.0% CLA
Initial body weight (g)	102 ± 1	101 ± 1	102 ± 1
Final body weight (g)	170 ± 2	166 ± 3	162 ± 4
Food intake (g/day)	19.1 ± 0.2	18.9 ± 0.3	18.6 ± 0.3
Tissue weight (g/100 g body weight)			
Liver	4.17 ± 0.09 <sup>a</sup>	4.11 ± 0.09 <sup>a,b</sup>	4.54 ± 0.07 <sup>b</sup>
Kidney	0.85 ± 0.03	0.86 ± 0.03	0.87 ± 0.05
Perirenal adipose tissue	1.41 ± 0.07 <sup>a</sup>	1.09 ± 0.09 <sup>a,b</sup>	0.97 ± 0.14 <sup>b</sup>
Heart	0.40 ± 0.02	0.34 ± 0.04	0.34 ± 0.04
Lung	0.48 ± 0.02	0.52 ± 0.02	0.49 ± 0.01
Spleen	0.22 ± 0.01	0.22 ± 0.01	0.25 ± 0.02
Brain	0.66 ± 0.02	0.70 ± 0.01	0.70 ± 0.01
Testis	0.96 ± 0.04	0.87 ± 0.10	1.00 ± 0.03

<sup>a</sup>Mean ± SE of 5 rats. Control group received 1.0% linoleic acid; 0.5% CLA group, 0.5% each of linoleic and CLA; and 1.0% CLA group, 1.0% CLA, respectively. Values without a common superscript letter (a,b) are significantly different at  $P < 0.05$ . For abbreviation see Table 1.

cyte total lipids is shown in Table 3. There was a dose-dependent reduction by dietary CLA of all n-6 PUFA, 18:2, 20:3, 20:4 and 22:4 in PBC lipids, while there was no difference in the proportion of n-3 PUFA, 22:6 among the groups. A clearer change in these n-6 PUFA was shown in splenic lymphocyte total lipids, and the reduction of 20:4n-6 was significant on a 1.0% CLA diet. Docosahexaenoic acid also tended to decrease with dietary CLA. The decreasing trend of all PUFA in CLA-fed rats was mainly attributable to a moderate increase in major saturated fatty acids, and oleic acid tended to decrease similar to PUFA (data not shown).

**Serum thiobarbituric acid value.** The concentration of thiobarbituric acid-reactive substance in serum was not modified by dietary CLA, and the values were within 4.1 to 5.5 ng/mL serum in all groups of rats.

**Serum Ig levels.** As shown in Figure 5, CLA increased the concentration of IgA, IgG and IgM, while decreasing that of IgE in serum. The difference between the control and 1.0% CLA groups was significant in these Ig.

**Ig levels in spleen and MLN lymphocytes.** Table 4 shows

the Ig levels in the medium of rat spleen and MLN lymphocytes cultured for 72 h with or without lipopolysaccharide. Irrespective of the presence or absence of lipopolysaccharide, CLA showed no detectable effects on the Ig levels in spleen lymphocytes except for those of IgM after incubation with lipopolysaccharide, where CLA increased it in a dose-dependent manner. Under the similar situation, CLA increased the concentration of IgA, IgG, and IgM in MLN lymphocytes. The magnitude of the increase was particularly marked at the dietary CLA level of 1.0%. In contrast, there was a significant reduction of the IgE level when the cells from rats fed a 1% CLA diet were incubated with lipopolysaccharide in comparison with the control. A similar response to CLA also was observed even when these cells were incubated for 24 h (data not shown).

**Subsets of MLN lymphocytes.** The proportion of T-lymphocyte populations of MLN was analyzed as CD4<sup>+</sup> and CD8<sup>+</sup> subsets. There were no effects of CLA on their relative proportions (CD4<sup>+</sup>/CD8<sup>+</sup> ratio; 2.6 ± 0.3, 2.4 ± 0.2, and 2.8 ± 0.1 for the control, 0.5% CLA, and 1.0% CLA, respectively).

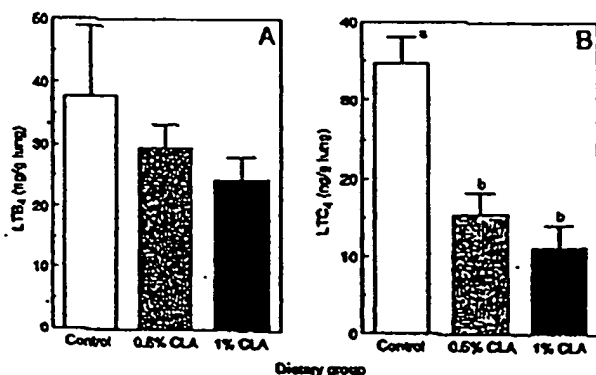


FIG. 3. Effect of dietary CLA on the concentration of lung (A) LTB<sub>4</sub> and (B) LTC<sub>4</sub>. Mean ± SE of five rats. Values without a common letter are significantly different at  $P < 0.05$ . For abbreviations see Figures 1 and 2.

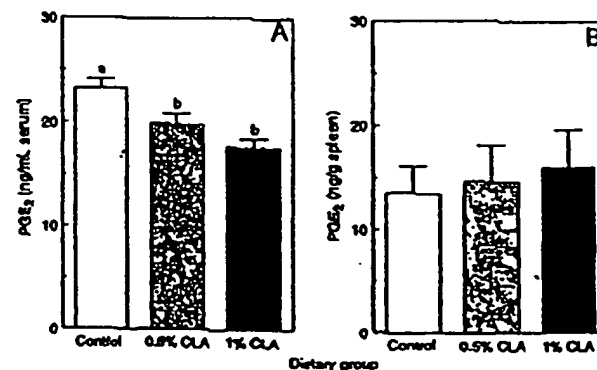


FIG. 4. Effect of dietary CLA on the concentration of (A) serum and (B) spleen prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). Mean ± SE of five rats. Values without a common letter are significantly different at  $P < 0.05$ . For other abbreviation see Figure 1.

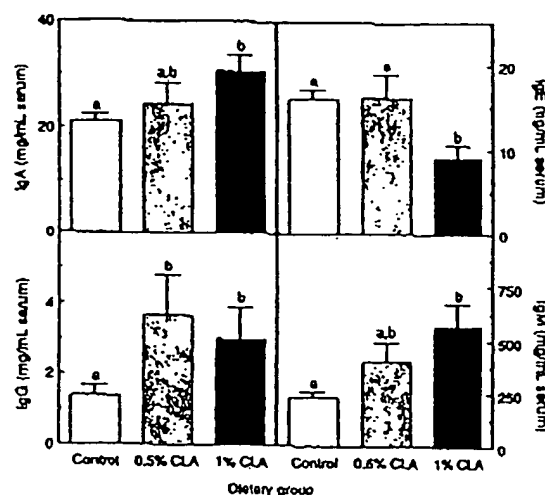
**TABLE 3**  
Effects of CLA on Polyunsaturated Fatty Acid Compositions  
of Peritoneal Exudate Cells and Spleen Lymphocyte  
Total Lipids of Rats<sup>a</sup>

Cells and fatty acid	Group		
	Control	0.5% CLA	1.0% CLA
		(wt%)	
Peritoneal exudate cells			
18:2n-6	5.5	5.3	4.2
20:3n-6	0.8	0.7	n.d.
20:4n-6	12.7	11.3	9.0
22:4n-6	5.6	5.3	4.2
22:6n-3	0.6	0.6	0.5
CLA			
9c,11c,9c,11c	n.d.	0.1	0.2
10c,12c	n.d.	0.2	0.2
Spleen lymphocytes			
18:2n-6	12.2 ± 0.8	10.4 ± 0.9	9.3 ± 0.9
20:3n-6	1.6 ± 0.2	1.3 ± 0.3	0.9 ± 0.1
20:4n-6	20.2 ± 0.8 <sup>a</sup>	15.4 ± 1.3 <sup>a,b</sup>	14.7 ± 1.7 <sup>b</sup>
22:4n-6	2.5 ± 0.1	2.0 ± 0.2	1.9 ± 0.2
22:6n-3	1.2 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
CLA			
9c,11c,9c,11c	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
10c,12c	n.d.	0.2 ± 0.0	0.2 ± 0.0

<sup>a</sup>Values are means of two pooled samples from two and three rats each for the exudate cells, and means ± SE of three, five, and five rats for control, 0.5% CLA, and 1.0% CLA, respectively. Values without a common superscript letter (a,b) are significantly different at  $P < 0.05$ ; n.d., not detected. For other abbreviation see Table 1.

## DISCUSSION

The pathway from linoleate to arachidonate and then eicosanoids is crucial to a range of metabolic diseases (28,29). Food allergy is one such disorder, and it is known that some eicosanoids are involved as chemical mediators in the manifestation of clinical symptoms of hypersensitivity (12,13). The inhibitors of LT production have now been clinically adopted (30,31). However, less is known of the effect that food components exert on this process. Although several food components have been shown to reduce eicosanoid production



**FIG. 5.** Effect of dietary CLA on the concentration of serum immunoglobulins (Ig). Mean ± SE of five rats. Values without a common letter (a,b) are significantly different at  $P < 0.05$ . For abbreviation see Figure 1.

*in vitro*, in most cases it is practically unsatisfactory because of the limited efficacy (21,22). The results of the present study showed that CLA effectively controlled the production of LTB<sub>4</sub>, LTC<sub>4</sub>, and PGE<sub>2</sub>. CLA significantly reduced LTC<sub>4</sub> production in the lung but not in the spleen. A similar tissue-specific reduction of LTC<sub>4</sub> was observed in rats given sesamin and α-tocopherol simultaneously, while in the spleen LTB<sub>4</sub> but not LTC<sub>4</sub> was reduced (21,22). These observations suggest a complex interaction between dietary fat and antioxidants in the LT-producing system.

Numbers of animal studies showed that dietary PUFA effectively modify the production of eicosanoids, and there is an interaction between n-6 and n-3 PUFA (32). PUFA of the n-3 family suppress the production of eicosanoids from arachidonic acid and exert a substantial suppressing effect on carcinogenesis in breast and colon (33,34). However, the anticarcinogenic effect of n-3 PUFA is far less than that of CLA (2-4). Eicosanoid production is known to be dependent on

**TABLE 4**  
Effects of CLA on the Immunoglobulin Production in Splenic and Mesenteric Lymph Node Lymphocytes of Rats<sup>a</sup>

Immunoglobulin	Without lipopolysaccharide			With lipopolysaccharide		
	Control	0.5% CLA	1% CLA	Control	0.5% CLA	1% CLA
Spleen (ng/mL)						
IgA	3.75 ± 1.23	4.83 ± 0.99	3.78 ± 0.96	9.74 ± 2.45	13.6 ± 3.27	8.30 ± 2.50
IgG	51.0 ± 4.6	53.8 ± 2.3	61.5 ± 2.8	68.1 ± 2.4	71.9 ± 1.9	74.4 ± 1.9
IgM	223 ± 22	228 ± 6	246 ± 9	311 ± 9 <sup>A</sup>	348 ± 8 <sup>B</sup>	394 ± 6 <sup>C</sup>
IgE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mesenteric lymph node (ng/mL)						
IgA	1.65 ± 0.13 <sup>A</sup>	4.78 ± 1.77 <sup>B</sup>	5.05 ± 0.10 <sup>B</sup>	2.91 ± 0.23 <sup>A</sup>	8.72 ± 0.90 <sup>C</sup>	22.3 ± 0.7 <sup>C</sup>
IgG	n.d.	3.08 ± 0.69 <sup>A</sup>	28.1 ± 4.38 <sup>B</sup>	n.d.	4.64 ± 0.11 <sup>A</sup>	31.9 ± 4.1 <sup>B</sup>
IgM	1.86 ± 0.34 <sup>A</sup>	4.74 ± 0.50 <sup>A</sup>	96.6 ± 13.4 <sup>B</sup>	2.85 ± 0.44 <sup>A</sup>	6.36 ± 0.48 <sup>B</sup>	122 ± 9 <sup>C</sup>
IgE	3.81 ± 0.32	4.02 ± 0.33	3.64 ± 0.47	4.81 ± 0.17 <sup>A</sup>	4.52 ± 0.29 <sup>A</sup>	3.74 ± 0.21 <sup>B</sup>

<sup>a</sup>Means ± SE of five rats. Values without a common superscript letter (A,B,C,a,b,c) are significantly different at  $P < 0.05$ . The lymphocytes were incubated with or without lipopolysaccharide for 72 h, and the concentration of immunoglobulins (Ig) in the supernatant was measured; n.d., not detected.

the substrate availability (35). CLA reduced the proportion of n-6 PUFA including arachidonic acid in the immune cells as observed in the liver and other tissues (8,14). Because of the limited availability of PEC samples for fatty acid analysis, they were analyzed as two pooled samples from two and three rats each. Though the number of analysis may not permit us to draw a definite conclusion, it seems likely that fatty acid composition of PEC also responded similarly as in spleen lymphocytes. This reduction was at least responsible for the reduced production of LT and PG in these cells. CLA may affect metabolic interconversion of fatty acids in the liver that may ultimately result in modified fatty acid composition and arachidonate-derived eicosanoid production in extrahepatic tissue (14). However, more direct participation of the metabolites of CLA cannot be ruled out (9,36). Therefore, the present study added possible usefulness of CLA for controlling the allergic reaction caused by food. Since the effect of CLA on Ig production differed between MLN lymphocytes and spleen lymphocytes, the analysis of the fatty acid composition of the former cells may provide a clue to understanding the mechanism of action.

In contrast to the eicosanoid production, the level of histamine released from PEC, which reflects the mast cell degranulation by a receptor-independent pathway, apparently was not modified by CLA and more directly the fatty acid composition of membrane phospholipids. Engels *et al.* (37)

observed that the type of dietary fats and thus the change in the fatty acid composition of mast cell phospholipids did not influence the cell degranulation process. CLA is reported to be incorporated into triglyceride more preferably than phospholipids in tumor cells (7). Thus, CLA may not substantially influence the fatty acid composition of membrane phospholipids and hence, the structure and function of the membrane. In such a situation, the degranulation of the mast cells may not be modified largely.

An interesting observation is that CLA regulates the Ig production class specifically. Food allergy reaction is initiated by the production of allergen-specific IgE (12,13). IgA, in contrast, serves as an antiallergic factor by interfering with the intestinal absorption of allergen, and IgG also works as an antiallergic factor through the competition with binding of allergen to the receptor on the surface of the target cells such as mast cells and basophiles (12,13). CLA increased the production of IgA and IgG, while reducing that of IgE in lymphocytes, in particular MLN lymphocytes irrespective of the presence or absence of lipopolysaccharide, a cell activator. The response of splenic lymphocytes to CLA was less clear except for a slight but significant increase in IgM after lipopolysaccharide activation. However, the response pattern similar to MLN lymphocytes was observed in serum, indicating that CLA can modify the Ig level preferably even on a whole-body basis. Bile acids (24) and unsaturated fatty acids (25) also regulate antibody production class specifically, but in a manner contrasted from that of CLA. These compounds may promote the allergic response through an increase in IgE production and a reduction in IgA and IgG production. It is

plausible that the production of IgE and of IgA and IgG are at least reciprocally regulated. Thus, in addition to the favorable effect on the eicosanoid production, CLA was expected to mitigate the food allergic reaction.

The amounts of CLA ingested by rats of the present study corresponded to approximately 30 and 60 mg/100 g body weight for 0.5 and 1.0% CLA diets, respectively. These amounts were pharmacological when extrapolated to human, 18 and 36 g/60 kg body weight/day. However, as in the case of weight reduction in man, approximately 3 g/d for 2 to 3 mon, a prolonged ingestion may produce a favorable effect even at a lower dose. A long-term trial with a lower dietary level of CLA merits further study.

In conclusion, CLA produced a situation favorable for mitigation of food allergic reaction. Since the effect was seen at a dietary level as low as 0.5 or 1.0%, it is likely that CLA can strongly regulate multiple metabolic processes. Thus, the clinical application of CLA is warranted. Studies with immunized animals will provide more direct information regarding this issue.

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